

Immunoglobulin G Antibodies in Human Vaginal Secretions after Parenteral Vaccination

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The induction of antibodies in vaginal secretions by systemic (intramuscular) immunization in humans was investigated by using the tetanus toxoid vaccine. Five women, 30 to 40 years old, were injected with a currently used dose of toxoid (40 IU), and serum, saliva, and vaginal secretion samples were collected on day 0 and on day 6 or day 10. All of these subjects had been previously vaccinated at least 5 years before; four were in good health, whereas one suffered from AIDS in clinical category B3. In most cases, analysis of specific antibodies in the vaginal wash showed a dramatic rise after boosting. These antibodies were primarily of the immunoglobulin G (IgG) isotype. The specific activity (ratio of antibody titer to IgG concentration) was shown to increase after the booster injection, irrespective of variations in the IgG level during the menstrual cycle. Comparison between serum and genital antibodies showed no difference in terms of both specific activity and level of avidity. These results demonstrate that parenteral injections can induce a systemic-derived antibody release in the vaginal fluid. Hence, systemic vaccinations can be efficient at the genital level and thus could reinforce or even replace a local vaccine.

Antibodies in secretions represent the first immune barrier against the entry of pathogens into the body throughout mucosae (6, 9, 10, 19, 20). Most of these antibodies consist of immunoglobulin A (IgA) polymers actively transported through epithelial cells after binding to the transmembrane secretory component (SC) (poly-Ig receptor). Secretory IgA (SIgA) is released into the lumen, where it remains bound to the SC (3). A role of SIgA antibodies seems to favor adherence of pathogens to mucus and thus their conveyance by the mucus stream. The presence of these antibodies in secretions is not correlated with the serum antibody level, and their induction by mucosal vaccination may be difficult. In vaginal secretions, the additional presence of significant levels of IgG has been reported (7, 8, 13, 28, 31–33). This IgG might originate from local plasma cells (2, 4, 14, 15, 30) and/or transude from the circulation, as suggested by the low number of these cells in the mucosa (2, 4). This release of serum antibodies in vaginal secretions might be of interest for vaccinations against AIDS and other sexually transmitted diseases.

In a previous study, our group has shown that antibodies to the human immunodeficiency virus (HIV) antigens in the vaginal fluid of infected patients are mainly of the IgG isotype (16). The contrast with the low antibody titer of SIgA suggested that these IgG antibodies were not locally produced but originated from the serum. This hypothesis led us to investigate the induction of antibodies in genital secretions by a conventional systemic route of vaccination. We show here that an intramuscular injection of a classical vaccine (tetanus toxoid) can induce IgG antibodies in the vaginal secretions.

MATERIALS AND METHODS

Subjects. Informed volunteers, 30 to 40 years old, received intramuscularly one vaccinating dose (≥ 40 IU) of purified tetanus toxoid, with aluminum hydroxide (≤ 1.25 mg of aluminum) as adjuvant (Vaccin tétanique; Pasteur-Mérieux, Marcy-

l'Etoile, France), more than 5 years after their previous vaccine injection. One subject (no. 5) suffered from AIDS in the clinical category B3 according to the 1993 classification of the Centers for Disease Control. Serum samples, vaginal secretions, and saliva samples were collected before (day 0) and after (day 6 or day 10) injection. The vaginal fluids were collected by washing with 3 ml of phosphate-buffered saline (PBS) and centrifuged. The final supernatant corresponded to approximately a 10-fold dilution of the original secretion. Except for subject 5, who suffered from amenorrhea, the vaccine injection was carried out during the follicular phase to avoid interference with menstruation. Hence, subject 1 was injected on day 6 of the menstrual cycle, subjects 2 and 4 were injected on day 8, and subject 3 was injected on day 11. No local infection was clinically apparent in any woman, and both washing and collection procedures were atraumatic. Resting mixed salivas were collected before meal time for 10 min and centrifuged. All samples were aliquoted and stored at -80°C until use.

Levels of Igs and albumin. Serum IgG and IgA were measured by a current nephelometric method (Behring, Marburg, Germany). IgG and IgA concentrations in secretions were measured by symmetrical sandwich enzyme-linked immunosorbent assay (ELISA) with affinity-purified sheep antibodies to human γ chain and α chain, respectively. Both antibodies were prepared and peroxidase labeled in our laboratory (11). The plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with $3\text{ }\mu\text{g}$ of unlabeled antibodies per ml ($100\text{ }\mu\text{l}$ per well), blocked with 2% skim milk powder in PBS for 2 h at 37°C , and washed three times with PBS containing 0.05% Tween 20. Two dilutions of each sample in the milk solution were applied in duplicate for 1 h at 37°C . After three washes, the corresponding labeled antibodies ($3\text{ }\mu\text{g/ml}$) were added, left for 1 h at 37°C , and washed. The peroxidase activity was revealed with *o*-phenylenediamine (Sigma, St. Louis, Mo.) and read at 492 nm with a Titertek Multiskan spectrophotometer (Flow Laboratories, Glasgow, United Kingdom). Dilutions of the secretions were adjusted to absorbances (units of optical density at 492 nm [OD_{492}]) similar to those of the corresponding serum dilutions measured in the same plate. Dilutions of a

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pool of normal sera served as a control curve. SIgA levels were measured by asymmetrical sandwich ELISA with coated antibodies to α chain and peroxidase-labeled antibodies to SC (11). Dilutions of SIgA, purified from human colostrum (12), served as a control curve. Albumin levels were measured by a similar method, using coated rabbit antibodies and peroxidase-labeled rabbit antibodies to human serum albumin. Dilutions of purified human albumin (Sigma) served as a control curve.

Antitoxins. Isotype-specific antibodies were measured by indirect ELISA, using plates coated with 2.5 μ g of pure tetanus toxoid per ml (100 μ l per well), kindly provided by B. Bizzini (Institut Pasteur, Paris, France). After saturation of the plates with the milk solution, serum dilutions were incubated for 1 h at 37°C, and bound antitoxins were revealed with peroxidase-labeled antibodies to γ chain or α chain. The IgG antitoxins in serum and secretions were compared by simultaneous measurements of samples adjusted to the same concentration of total IgG. Each assay was carried out in duplicate at two dilutions and compared with five dilutions of a standard serum containing 50 IU/ml (provided by B. Bizzini). The specific activity of IgG (in international units per milligram) was determined by the ratio of IgG antibodies to total IgG. Serum IgA and IgM antibodies were measured by the same method, using affinity-purified sheep antibodies to human μ or α chain (3 μ g/ml) prepared and labeled in our laboratory. In the absence of standards for these isotypes, IgA and IgM antitoxins were expressed as dilutions giving 0.2 OD₄₉₂.

SIg antitoxins (primarily SIgA) were detected in secretions after absorption of IgG and compared with spontaneous salivary antibodies against *Streptococcus sobrinus*, a cariogenic microorganism. Diluted secretions were incubated at 37°C for 1 h with a sufficient volume of sheep antibodies to Fc γ , insolubilized with cyanogen bromide onto Sepharose 4B beads (Pharmacia, Uppsala, Sweden), with the volume of the swollen gel being taken into account for the final dilution of the sample. Half of the wells in each plate were coated with tetanus toxoid, and the other half were coated with 0.1 μ g of cell wall carbohydrates, purified from *S. sobrinus* and kindly provided by H. Hocini of our laboratory, per ml. Dilutions were incubated overnight at 4°C, and the secretory antibodies were detected with peroxidase-labeled antibodies to human SC, as previously described (11). Because of their low levels, the secretory antibodies were expressed as OD₄₉₂ values at a 10-fold dilution.

Avidity index. The avidities of IgG antitoxins in serum and genital secretions were compared by the method of Pullen et al. (27) with slight modifications. Toxoid-coated plates were incubated for 1 h at 37°C with a single dilution of each sample, chosen to give approximately 1 OD₄₉₂ after reaction with the labeled antibodies to human γ chain. After several washings, 100- μ l portions of appropriate molarities of ammonium thiocyanate in PBS were added in duplicate and left for 30 min at room temperature. After further washings, the usual ELISA procedure was carried out. Results were expressed as percentages of the OD₄₉₂ values obtained with control wells incubated with the same dilutions of thiocyanate before incubation with the sample.

RESULTS

Serum antibody responses. In most subjects, a high level of IgG antitoxins on day 0 revealed a very long-lasting response to previous vaccinations carried out more than 5 years before (Table 1). After injection, various types of responses were observed, depending on the level of antitoxins on day 0 as well as on the clinical background (Table 1). Normal subjects 2, 3,

TABLE 1. Comparison of specific activities of IgG antitoxins in serum and vaginal washes before (day 0) and after (day 6 or 10) vaccine injection

Subject	Day	Serum		Vaginal wash ^a		Sp act ratio, vaginal wash ^b /serum
		IgG (mg/ml)	IgG antitoxins (IU/ml)	IgG (μ g/ml)	IgG antitoxins (mIU/ml)	
1	0	19	3.2	35	5.5	0.96
	10	16	11	100	42	0.60
2	0	18	<0.1	30	<0.5	ND ^c
	10	17	10	130	32	0.41
3	0	20	0.3	47	0.75	0.94
	10	19	4.3	370	59	0.70
4	0	20	0.63	41	0.74	0.57
	10	20	4.6	370	59	0.76
5 ^d	0	27	0.75	330	10	1.16
	6	22	1.7	130	5	0.58

^a Corresponding to a ca. 1:10 dilution of the vaginal fluid.

^b Calculated from crude results.

^c ND, not determined.

^d AIDS patient, category B3.

and 4, who had low initial levels, exhibited strong IgG antibody responses on day 10, whereas the rise was low for subject 1, who had a high initial level, and for the HIV-seropositive subject. In contrast to IgG antitoxins, which were assayed at high serum dilutions (median dilution, 1/500,000 for 1 OD₄₉₂ antitoxins of the IgA and IgM isotypes were detected only at low dilutions, although the sensitivities of detection were similar for the three isotypes. For IgA antitoxins, the serum dilution giving 0.2 OD₄₉₂ ranged from 1/100 to 1/200 on day 0 in the four normal subjects and was 1/400 on day 10. In the same subjects, the apparent titers of IgM antitoxins were all 1/40 and did not increase after boosting. The IgA antitoxin titer of the HIV-positive woman was 1/50 on day 0 and 1/100 on day 6, whereas the IgM titer remained at 1/40 during this period.

Antibodies of the IgG isotype in vaginal secretions. Like for the sera, a significant level of IgG antitoxins was detected in the genital secretions of most subjects on day 0. After injection, a dramatic rise was observed in subjects 2, 3, and 4, who initially showed low levels of antitoxin (Table 1), whereas this increase was much lower in subject 1. In the AIDS patient, the response on day 6 was absent, with the apparent decrease being parallel to that of total IgG.

To confirm that the differences between results for days 0 and 10 were actually due to the booster injection in the normal women, the level of total IgG was correlated with the date of menstruation, and the ratios of IgG antitoxins (in international units) to IgG concentrations (in milligrams per milliliter) before and after the booster injection were compared. The level of IgG, as well as that of IgA, in the vaginal wash fluids was found to be clearly lower during the follicular phase (IgG ranged from 30 to 47 μ g/ml, and IgA ranged from 4 to 8 μ g/ml) than during the luteal phase (IgG ranged from 130 to 370 μ g/ml, and IgA ranged from 17 to 55 μ g/ml) of the cycle. Nevertheless, the increase in total IgG could not explain the rise of IgG antitoxins, as shown by a simultaneous increase of the specific activity after the booster injection (Fig. 1). Moreover, comparisons of the specific activities in secretions and in serum showed these values to be highly related to the secre-

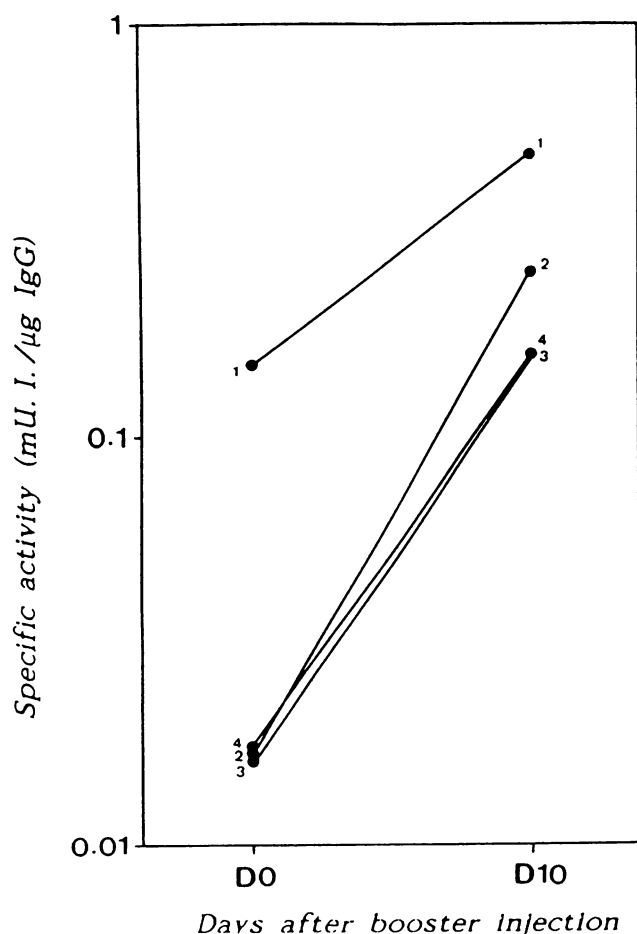


FIG. 1. Increase of specific activity of IgG antitoxins in vaginal washes after booster injection of HIV-negative women (subjects 1 to 4). U.I., international units.

tion/serum IgG ratios, which remained close to 1 after the injection, whatever the degree of antitoxin response (Table 1). Investigation of the functional avidity of IgG antitoxins by thiocyanate dissociation in subject 1 (Fig. 2A), who was selected for her high level of responsiveness, and in the AIDS patient (Fig. 2B) showed identical curves for serum and for vaginal secretions.

To investigate whether the secreted IgG only diffused from the serum, albumin in the vaginal washes was measured and its level was compared with that of IgG. In the normal subjects, the albumin level was found to be relatively low, ranging from 8 to 18 $\mu\text{g/ml}$ on day 0 and from 15 to 45 $\mu\text{g/ml}$ on day 10. This level was higher in subject 5 (78 $\mu\text{g/ml}$ on day 0 and 180 $\mu\text{g/ml}$ on day 6), paralleling the IgG variation. Furthermore, a positive correlation was found between albumin and IgG levels ($r = 0.83$; $P < 0.01$) for all subjects.

Secretory antibodies. After absorption of IgG, which can interfere in the ELISA by competition with antitoxins of the other isotypes, no significant SIg antitoxin activity was detected in genital secretions of most patients, with only subjects 3 and 4 showing a very low response on day 10 (0.16 and 0.17 OD_{492} , respectively). To ensure that this lack of activity was not due only to insufficient levels of SIgA in vaginal washes (ranging from 0.6 to 2.5 $\mu\text{g/ml}$ on day 0 and from 5.9 to 16 $\mu\text{g/ml}$ on day 10), antitoxins in saliva were also investigated (salivary SIgA

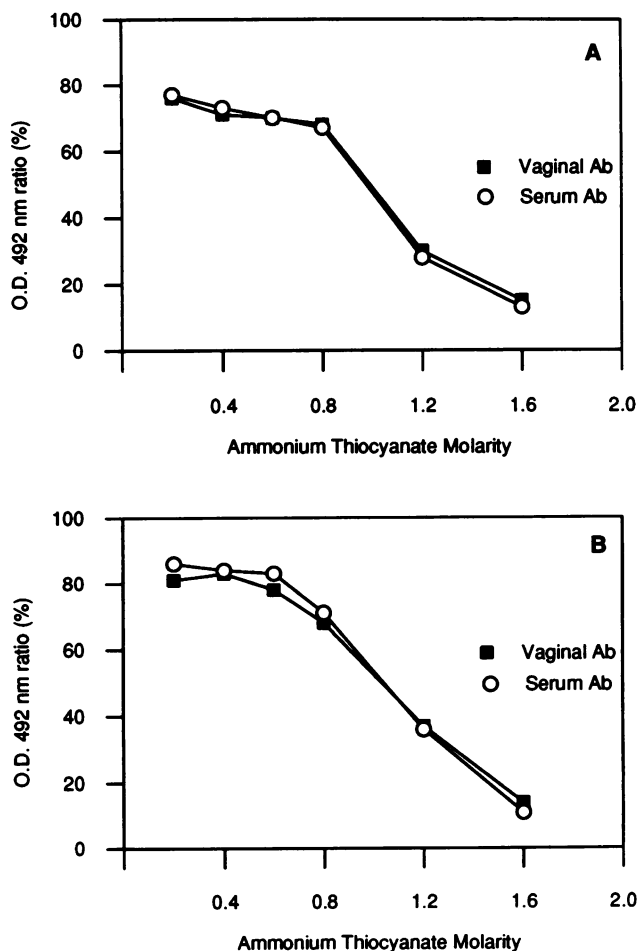


FIG. 2. Comparative avidity curves of IgG antitoxins in serum and in cervicovaginal fluid after vaccination. Samples were from subjects 1 (normal) (A) and 5 (HIV seropositive) (B). Ratios were drawn from the OD_{492} values obtained with wells incubated with ammonium thiocyanate after and before addition of the antitoxins.

ranged from 60 to 82 $\mu\text{g/ml}$ on day 0 and from 65 to 170 $\mu\text{g/ml}$ on day 10). Similarly, the initial levels of salivary SIg antitoxins were very low (ranging from 0.07 to 0.3 OD_{492}), and furthermore, no significant rise was observed after boosting (ranging from 0.1 to 0.32 OD_{492}). As an internal control, antibodies to cell wall carbohydrates were clearly positive in all salivas (ranging from 0.56 to 1.05 OD_{492}).

DISCUSSION

Investigating a possible induction of specific antibodies in vaginal secretions after parenteral injection of the antigen, we showed that the currently used tetanus toxoid vaccination leads to a substantial rise of local IgG antitoxins. These antibodies most likely have a systemic origin and can occur in the absence of specific SIg. The use of current methods of vaccination could thus be of major interest for prevention against sexually transmitted pathogens in humans, as a substitute for local immunization, when local immunization is not available, and/or as a complement to prolong the time of protection.

Human spontaneous genital antibodies of the IgG isotype are most often against the pathogens and alloantigens which reach the immune system via the genital route. Hence, it has

not been established whether the local-regional and the systemic immune systems are involved alone or simultaneously in these cases. Experimental investigations with rodents showed antibodies of the IgG isotype both in serum and in vaginal secretions after intravaginal immunization with purified antigens (21, 25) as well as with live microorganisms (5, 18). It has been shown that mice (25, 26, 29) and sheep (24) immunized by the subcutaneous route develop a high antibody response of the IgG isotype in the vagina. Similar results were obtained in monkeys immunized with native (22) or formalin-treated (17) simian immunodeficiency virus. For humans, it was also observed that systemic vaccination against killed polio vaccine can induce both serum and vaginal antibodies of the IgG isotype (23). Nevertheless, all these experimental studies focused mainly on IgA antibodies, and therefore the IgG responses were not extensively investigated.

We confirm here the presence of IgG in vaginal secretions. The levels varied from one sampling to another, as previously observed for all Ig classes in other secretions. The method of collection by washing could lead to a variable degree of dilution, as suggested by the correlation between IgG and albumin values. Furthermore, preliminary unpublished results showed a higher concentration of fluid in washes during the luteal phase than during the follicular phase. This difference is due to a rise of the fluid volume and could explain in part variations in IgG concentration according to the phase of the menstrual cycle in rats during estrus, where it is related to a stimulatory effect of estrogens (34). Similarly, in humans both IgG-positive cells, which might be nonimmunocytic cells having taken up IgG from the interstitial fluid (2), and IgG-positive glands were found to increase in the endometrium during the luteal phase (2).

In the vaginal washes of normal subjects, the IgG/total IgA ratio was close to that in serum (i.e., ~8) and differed significantly from the values (0.5 to 4) reported in previous studies (7, 8, 13, 28, 32, 33), but it was in agreement with the results of a more recent study (31). These differences seem to be related to a higher estimation of IgG in our study, probably because the samples were centrifuged immediately after collection instead of being stored frozen without preliminary cell removal. Indeed, this method prevents the release of cellular proteases during thawing and thus prevents degradation of IgG and, to a lesser degree, of nonsecretory IgA. In contrast, SIgA is known to be resistant to most proteases. The IgG concentration found in our study was ca. 31-fold (median value) higher than that of SIgA in the vaginal secretions. Even if SIgA could have been underestimated by competitive inhibition with monomeric IgA during the ELISA, this ratio clearly differs from the ratio of IgA to IgG plasma cells in both cervical and endometrial mucosae (2, 14), but it is in full agreement with the large amount of extracellular IgG recently found in these tissues (4). From this latter study (4), it seems that both mucosal and serum-derived IgA polymers are actively translocated through the endometrium epithelial cells to form endoluminal SIgA. Provided that serum-derived albumin was not degraded and/or selectively reabsorbed, its low level in the vaginal secretions, compared with that of IgG, suggests that passive transudation alone cannot explain the high level of IgG. Local synthesis and/or transport mechanisms, e.g., via Fc γ receptors, must also be envisaged. The Ig transudation might be through the endometrium and cervix (4), since the squamous epithelium of the vagina seems unfavorable for diffusion (15).

The levels of IgG antitoxins were related to the corresponding levels of total IgG in order to avoid interference with intrinsic and sampling variations. Taking into account the

standard error of each measurement and the fact that the ratios were drawn from results obtained at very different dilutions, the specific activity in secretions was considered to be highly similar to that found in serum (Table 1). Moreover, investigation of their relative avidities confirms that IgG antitoxins in genital secretions were both of high affinity and identical in nature to their serum counterparts. A significant involvement of the local immune system at the early phase of boosting (day 10), as well as at the late phase of the previous vaccination (day 0), seems to be unlikely, since the level of SIg antitoxins was low enough in genital secretions to be explained solely by the active transport of serum-derived polymers across epithelial cells, as proposed by Brandtzaeg et al. (4). In addition, investigation of the saliva did not show a significant SIg antitoxin increase at another mucosal site, whereas the control level of spontaneous SIg antibodies to cariogenic bacteria was much higher. Therefore, our overall results show that even if a local synthesis can be suspected in the IgG antitoxin production, the corresponding plasma cells must be related to the systemic instead of the mucosal immune system.

Systemic-derived immunity in human genital secretions reinforces the potential interest in vaccines given by the parenteral route in prevention of sexually transmitted diseases. Indeed, we show here that an intramuscular immunization can locally induce for a long period of time high levels of antibodies, which might be protective against the corresponding pathogen, given the efficacy of the injected antigen. Despite the higher activity of SIgA compared with IgG against pathogens (1), the possibility of inducing protective antibodies by current procedures before development of true secretory vaccines seems to be of major interest. Antibodies in secretions can play a key role against the pathogens which remain in the genital area, by increasing their clearance with the mucus and by inhibiting their attachment to the mucosa. These antibodies could thus decrease, or even suppress, the entry of pathogens through the mucosa and avoid overloading of the systemic defenses. Although isotype-associated effector functions of the Fc γ domains might produce adverse effects, it is likely that IgG antibodies exert mucosal protection in cervicovaginal secretions. Such mechanisms could be useful against sexual transmission of HIV by preventing the virus from reaching the systemic immune system.

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